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(54) Title: EXPRESSION OF THE BOVINE PARAINFLUENZA VIRUS TYPE 3 HEMAGGLUTININ/NEURAMINIDASE (HN) GLYCOPROTEIN IN TWO HETEROLOGOUS SYSTEMS

(57) Abstract

New vaccines to combat respiratory diseases in cattle are produced by inserting the cDNA that codes for the bovine parainfluenza virus hemagglutinin/neuraminidase (HN) glycoprotein into bovine herpesvirus type 1 (BHV-1) or into baculovirus, such that the protein is expressed by the respective viruses.

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Expression of th Bovin Parainfluenza Virus Type 3 Hemagglutinin/Neuraminidase (HN) Glycoprotein in Two Heterologous Systems

BACKGROUND OF THE INVENTION

Field of invention

This invention relates to the field of Bovine Parainfluenza Virus Type 3, and vaccines for the treatment thereof.

Information Disclosure

U.S. Patent 4,847,081, John M.Rice , issued July 11, 1989.

U.S. Patent 4,703,011, Malon and Saul Kit, issued October 27, 1987.

U.S. Patent 4,992,051, Malon and Saul Kit, issued February 12, 1991.

R.W. Morgan, *et al.*, *Avian Diseases*, 36:858-870 (1992),Y. Sakai, *et al.*, *J Virol*, 63:3661-8 (1989),T. Shioda, *et al.*, *Virology*, 162:388-96 (1988),S. Suzu, *et al.*, *Nucleic Acids Res*, 15:2945-58 (1987),K.L. Van Wyke Coelingh, *et al.*, *Virology*, 160:465-472 (1987).

Background

Bovine parainfluenza virus-3 (BPIV-3) belongs to the genus *Paramyxovirus* of the *Paramyxoviridae*. Virions are pleomorphic, enveloped particles which contain a nucleocapsid that incorporates a single stranded RNA genome. The genome serves as a template for transcription of six unique structural proteins, nucleoprotein (NP), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large protein (L).

The HN and F proteins project as spike-like structures from the virion surface, and are also expressed on the surface of infected cells. The HN protein is strongly hydrophobic at the amino terminus and this domain is important in anchoring the protein into the virion envelope and host-cell membrane. A second domain, closer to the C-terminus, mediates attachment of the virion to sialic acid-containing host cell receptors during the initial phase of the infection (R.M. Chanock, *et al.*, Virology, B. Fields, ed. (1990)).

The hemagglutination reaction, which is widely used in sero-diagnosis and experimental laboratory studies, is also a manifestation of this type of interaction and is the reason that this glycoprotein is designated as a hemagglutinin. Later in infection, the HN protein also cleaves sialic acid residues from the virion surface by virtue of its neuraminidase activity.

After attachment to the cell th virus has to deliver the nucleocapsid into the cytoplasm and this is mediated by the second surface glycoprotein, the F protein. This occurs with the fusion f the viral lipoprotein envelope with the lipoprotein surface of the host cell. The F glycoprotein is also expressed on the surface of infected cells which allows fusion of contiguous infected and uninfected cells to occur, leading to syncytium formation and an extension of the infection. The crucial role which these two proteins have in the infective process, and their presence on the surface of infected cells, makes them obvious targets for the immune system and studies have shown that animals which have recovered from infection have neutralizing antibody to both of these glycoproteins.

The importance of BPIV-3 as a pathogen is its association with bovine respiratory disease complex (BRDC). BRDC is the major cause of morbidity and mortality in cattle leading to extensive economic losses. The commonly held view is that viruses, bacteria and stress interact to cause the condition. The viruses most often implicated are bovine herpesvirus type-1 (BHV-1), BPIV-3, bovine viral diarrhea virus (BVDV) and bovine respiratory syncytial virus. With stress, leading to an increased susceptibility to infection, viral pathogens are thought to cause initial damage to respiratory tract epithelium which then allows bacterial invasion to occur and a further exacerbation of clinical signs (R.J. Yancey, *J Dairy Sci*, 76:2418-36 (1993)).

It is often assumed that more than one viral agent is involved at any one time and indeed virus isolation and serological evidence in BRDC support that view. For this reason most viral vaccines developed to prevent this disease contain antigens for each of these viruses.

Although a wide variety of combination vaccines against the viral pathogens associated with BDRC are available, this disease complex remains a major source of economic loss in the cattle industry. Commonly used vaccine preparations consist of various combinations of inactivated viruses (IV) and modified live viruses (MLV), with each preparation striking a particular balance between safety and efficacy. In all cases, however, combination vaccines require preparation of the various viral vaccines separately, which adds considerably to production complexities and cost. In addition, differences in optimal storage and administration protocols for the various components of multiple virus vaccine preparations can lead to compromises in potency and stability of some of the components.

This invention will provid the user with an effective vaccine for prevention of the BPIV-3 and/or BHV-1 caused components of the BDRC complex.

SUMMARY OF THE INVENTION.

This inventi n comprises n w vaccines to combat respiratory diseases in cattle. The vaccines are produced by inserting the cDNA that cod s for the bovine parainfluenza virus hemagglutinin/neuraminidase (HN) glycoprotein into bovine herpesvirus type 1 (BHV-1) or into baculovirus, such that the protein is expressed by the respective viruses. The invention comprises a replicating nonpathogenic virus, comprised of a gene or gene combination encoding a Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in any appropriate locus of bovine herpesvirus type 1 (BHV-1), a virus where the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein is from bovine parainfluenza virus type 3 (BPIV-3), a virus where the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein is inserted into and expressed in the thymidine kinase locus of BHV-1. A vaccine for the prevention of diseases caused by bovine parainfluenza virus type-3, consisting of a replicating nonpathogenic virus containing a gene or gene combination that encodes a Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in any appropriate locus of bovine herpesvirus type 1 (BHV-1), a vaccine where the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein is from bovine parainfluenza virus type 3 (BPIV-3), a vaccine, where the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein is inserted into and expressed in the thymidine kinase locus of BHV-1. A vaccine, consisting of a gene or gene combination encoding a Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in the baculovirus expression system, a vaccine where the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein is from bovine parainfluenza virus type-3 (BPIV-3). Pharmaceutical compositions, carriers, diluents, adjuvants appropriate for the viral vaccine are also provided or understood by one skilled in the art.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Figure 1. Construction of shuttle vector for expression of the BPIV-3 HN gene (and other genes) in the BHV-1 tk locus.

Figure 2. Construction of a recombinant baculovirus transfer vector for expression of the BPIV-3 HN gene in the baculovirus expression system.

Figure 3. Immunoprecipitations of infected-cell lysates showing expression of BPIV-3 HN from BHV-1 and baculovirus.

Figure 4. Neuraminidase activity in the BHV-1 and baculovirus

recombinants.

ADDITIONAL DESCRIPTION OF THE INVENTION AND THE PREFERRED EMBODIMENTS

Definitions

All of the terms used below will be readily understood by one skilled in the art. In many places the name of the manufacturer of equipment or reagents are provided in parenthesis after the equipment or reagent is named. Commonly used terms, reagents and buffers such as "plasmids," "Klenow Fragments," "religating blunt ends", "Tris", and chelating buffers such as EDTA are referred to without further explanation.

In the descriptions of the construction of the compounds used in this invention, standard molecular biological techniques were used and are briefly named or described here. Detailed explanations of these techniques can be found in standard laboratory manuals such as F.M. Ausubel, *et al.*, (1993), and J. Sambrook, *et al.*, (1989).

Any abbreviations that would not necessarily be readily understood by one skilled in the art, such as"BV", "BT cells", "BDRC", "BPIV-3" and "BHV-1", are defined upon their first use, and are used thereafter, usually without further elaboration.

Compositions and Administrations - A pharmaceutically effective amount of the vaccine of the present invention can be employed along with a pharmaceutically acceptable carrier, diluent or adjuvant as a vaccine against BHV-1 and BPIV-3 in animals, such as bovine, sheep and goats.

Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include any physiological buffered medium, i.e., about pH 7.0 to 7.4, containing from about 2.5 to 15% serum which does not contain antibodies to BHV-1, i.e., is seronegative for BHV-1. Serum which does not contain gamma globulin is preferred to serum which contains gamma globulin. Examples of serum to be employed in the present invention include fetal calf serum, lamb serum, horse serum, swine serum, and goat serum. Serum protein such as porcine albumin or bovine serum albumin (hereinafter "BSA") in an amount of from about 0.5 to 3.0% can be employed as a substitute for the serum. However, it is desirable to avoid the use of foreign proteins in the carrier or diluent which will induce allergic responses in the animal being vaccinated. Examples of pharmaceutically acceptable adjuvants are those routinely used to potentiate non-replicating antigens, such as, oil in water, water in oil, saponins, and lipopolysaccharides (LPS), etc.

The virus may be diluted in any of the conventional stabilizing solutions containing phosphate buff r, glutamate, casitone, and sucrose or sorbose, r containing phosphate buffer, lactose, dextran and glutamate.

It is preferred that th vaccine viruses of the present invention be stored at a titer of at least 10^5 to 10^6 PFU/ml at -70°C to -90°C or in a lyophilized state at 2°C to 7°C. The lyophilized virus may be reconstituted for use with sterile distilled water or using an aqueous diluent containing preservatives such as gentamicin and amphotericin B or penicillin and streptomycin.

The useful dosage to be administered will vary depending upon the age, weight and species of the animal vaccinated and the mode of administration. A suitable dosage can be, for example, about $10^{4.5}$ to 10^7 PFU/animal, preferably about $10^{4.5}$ to $10^{5.5}$ PFU.

The vaccines of the present invention can be administered intranasally, intravaginally or intramuscularly. Intranasally is the preferred mode of administration for the BHV-1 component, and intramuscularly is the preferred mode of administration for the baculovirus component.

The Compounds and the Preparation of the Compounds of the Invention

The specific methods that we used to in the preparation the compounds of this invention are given below. These methods would enable anyone skilled in the art to reproduce the claimed invention. Alternative methods are available for a variety of these manipulations, including cloning, transformation, tissue culture and immunological detection, and are described in standard laboratory manuals (F. M. Ausubel, et al., (1993), J. Sambrook, et al., (1989)). These alternative methods would also allow reproduction of this invention by one skilled in the art

A cDNA clone of the BPI-3 HN gene can be constructed according to procedures well known in the art or by following the procedures described in Y. Sakai, et al., *J Virol*, 63:3661-8 (1989) and H. Shibuta, *et al.*, *Virology*, 155:688-96 (1986), incorporated by reference herein. This cDNA, available from the procedure described above or as obtained from Dr. Hiroshi Shibuta, Tokyo University, is inserted into shuttle vectors for recombination and expression in BHV-1 and baculovirus (BV). For introduction into BHV-1, the HN gene is inserted into the shuttle vector pHAS4DBXex-1 (the construction of which is described in this section). The insertion is made such that the HN gene disrupt the BHV-1 thymidine kinase gene. For baculovirus expression the HN gene is inserted into a standard, commercially available shuttl vector (PVL1392, Pharmingen, San Diego, CA) under the control of the polyhedron promoter. Following co-transfection of the shuttle

plasmids with their respective viral genomes, tk-negative BHV-1 and polyhedron-negative BV are isolated and screened for the presence of the HN gene by Southern hybridization and for the expression of HN by radioimmunoprecipitation.

Cells and Viruses. Bovine turbinate (BT) cells are available from the American Type Culture Collection (ATCC, Rockville, MD). The BT cells are grown in Dulbecco's modifed eagle medium (DMEM) supplemented with penicillin, streptomycin, non-essential amino acids and 10% horse serum. Thymidine kinase deficient rabbit skin (RabBU) cells were provided by Saul Kit and Malon Kit; however, any other thymidine kinase-deficient cell lines that support the growth of BHV-1 can be used to reproduce this invention. The tk deficient cells are grown in DMEM supplemented with penicillin, streptomycin, 10% fetal bovine serum and were periodically passaged in 100ug/ml 5-bromo-2'-deoxyuridine (BDUR, Sigma, St. Louis, MO). *Spodoptera frugiperda* (SF9) cells were propagated in Grace's Media (complete) supplemented with penicillin, streptomycin, fungizone and 10% fetal bovine serum. Tissue culture media, supplements and sera were all obtained from Gibco/BRL (Grand Island, NY). Bovine herpesvirus type 1 (BHV-1) strain "Iowa" can be obtained from the National Animal Disease Center of the USDA (Ames, Iowa). Other BHV-1 strains can be used. The SF-4 challenge strain of bovine parainfluenzavirus type 3 (BPIV-3) can be obtained from the National Veterinary Services Laboratory (Ames, IA). Efficacy of this invention could be shown, however, with any validated challenge model for BPIV-3 disease.

Viruses are grown and titered by standard methods that would be well known to those skilled in the art. Initial plaque isolations are carried out under 0.8% agarose, and plaque purifications were accomplished by limiting dilution in 96 well plates.

Molecular cloning and DNA purification techniques. The procedures used for the described molecular cloning techniques can be found in the standard references (F. M. Ausubel, et al., (1993), J. Sambrook, et al., (1989)). Restriction and modification enzymes can be purchased from any supplier, such as New England Biolabs (NEB, Beverly, MA), Gibco/BRL, TaKaRa (Madison, WI) or Boehringer Mannheim Biochemicals (BMB, Indianapolis, IN), and should be used according to the manufacturer's specifications. Recombinant plasmids are transformed into competent *E. coli*, such as strain DH5a.

Frozen competent cells can be purchased from Gibco/BRL or other suppliers. DNA fragments excised from agarose gels can be purified using Qiaex (Qiagen, Chatsworth, CA), or by other methods. Plasmid DNAs can be purified using Qiagen

columns (Qiagen) or by other methods. BHV-1 DNA is purified from infected BT cells on sodium iodid gradients essentially by th method described by Wallboomers and Ter Shegett (J.M. Walboomers, *et al.*, *Virology*, 74:256-258 (1976)), or for small-scale preparations by the "mini-prep" procedure as described by Engel, et al (J.P. Engel, *et al.*, *Virology*, 192:112-120 (1993)). DNA hybridizations of viral DNA restriction digests are carried out using the digoxygenin/anti-digoxygenin reagents supplied with the Genius Kit (BMB) or by other applicable methods.

Previously cloned DNA fragments. Plasmid pHAS4, is a 2.7kb subfragment of the BHV-1 HindIII "A" fragment (from strain Cooper) cloned into vector pUC18. This plasmid can be assembled according to procedures well known in the art or by following the procedures described in (J.E. Mayfield, *et al.*, *J Virol*, 47:259-264 (1983)), incorporated by reference herein. This SalI fragment contains the entire thymidine kinase (tk) gene, the BHV-1 homolog of the HSV-1 UL24 gene and a small portion of the BHV-1 glycoprotein H gene (L.J. Bello, *et al.*, *Virology*, 189:407-414 (1992), J.G. Jacobson, *et al.*, *J Virol*, 63:1839-1843 (1989), A.L. Meyer, *et al.*, *Biochim Biophys Acta*, 1090:267-9 (1991), J.C. Whitbeck, *et al.*, *Virology*, 200:263-270 (1994)). A 424bp deletion is made in the tk gene by digesting with BglII and XhoI and blunt-end ligating the filled-in ends. This manipulation regenerates the BglII site, and the resulting plasmid is designated pHAS4DBX.

Creation of a shuttle vector for expression of foreign genes in the BHV-1 tk locus. In order to express various different viral antigens in BHV-1, a shuttle vector, pHAS4DBXex-1 was created to allow recombination of the foreign genes into the viral thymidine kinase locus. The construction of this vector is detailed in FIGURE 1. Begin with pHAS4, a 2.7kb BHV-1 SalI fragment which contains the entire tk gene and flanking regions on either side. Eliminate the single HindIII restriction site present in pHAS4 (cloned into the pUC18 vector, a DNA cloning vector well known to those skilled in the art (J. Sambrook, *et al.*, (1989))) by digesting with HindIII, filling in the ends and religating. A 424bp deletion Is made in the tk gene by digesting with enzymes BglII and XhoI, filling in the sticky ends and religating the resulting blunt ends (this manipulation regenerated the BglII restriction site), resulting in plasmid pHAS4DBX. Into this BglII restriction site insert an expression cassette consisting of the Human Cytomegalovirus (HCMV) immediate early promoter and the polyadenylation signal from bovine growth hormone, which are separated by a unique HindIII restriction site for the insertion of foreign genes to be expressed (R.J. Brideau, *et al.*, *J Gen Virol*, 74:471-477 (1993)).

This shuttle vector is called pHAS4DBXex-1. As shown in FIGURE 1, the promoter/polyadenylation signal cassette in this vector is inserted in the opposite transcriptional orientation to that of the original tk gene.

The HN gen was cloned MR2-9tr from strain MR (Y. Sakai, *et al.*, *J Virol*, 63:3661-8 (1989), H. Shibuta, *et al.*, *Virology*, 155:688-96 (198)),. This cDNA contains the complete HN mRNA cloned into an Okayama and Berg-type cloning vector (H. Okayama, *et al.*, *Molec Cell Biol*, 2:161-170 (1982)). This HN gene clone can be obtained or constructed according to procedures well known in the art or by following the procedures described in Y. Sakai, *et al.*, *Nucleic Acids Res*, 15:2927-44 (1987), incorporated by reference herein.

Generation of recombinant BHV-1 and baculovirus. To obtain recombinant BHV-1, unit length viral DNA and plasmids containing the sequences of interest are cotransfected into BT cells by the calcium phosphate method as described by Graham and Van der Eb (R.L. Graham, *et al.*, *Virology*, 52:456-467 (1973)), and as modified by Lawrence, *et. al.* (W.C. Lawrence, *et al.*, *J Virol*, 60:405-14 (1986)). Prior to cotransfection, the shuttle plasmids are linearized by digestion with Sse8387I and the resulting linear fragments are purified away from the enzyme using the Qiaex glass matrix (Qiagen) according to manufacturer supplied methods. Tk-negative viruses arising from the cotransfections are selected by two passages on RabBU cells in the presence of 100ug/ml BDUR. Selected BDUR-resistant viruses are then plaque purified three times on BT cells (in the absence of BDUR) by limiting dilution.

The BaculoGold system (Pharmingen, San Diego, CA) can be utilized for the generation of recombinant baculoviruses (BV), using protocols provided by the manufacturer. In this system, the shuttle plasmid carrying the foreign gene of interest is cotransfected with a defective genome of AcNPV such that only those genomes rescued by the recombination of flanking sequences on the shuttle plasmid will be viable in wild-type cells. Thus viral progeny from the transfection need only be plaque purified and screened for expression of the gene of interest.

Metabolic labelling of infected cells. For immunoprecipitations (as described later in this section) cells can be infected with BHV-1 or BPIV-3 and SF9 cells infected with recombinant BV at a multiplicity of infection of 5-10. For the BT cell infections the normal media is replaced with media containing 10% cold methionine and 100uCi/ml ^{35}S-Methionine (50 mCI/mMol, Amersham, Arlington Hights, IL) at 8 hours post-infection, and the cells allowed to label for another 24 hours. For the SF9 infected cells, the media is replaced with methionine-free Grace's media

containing 100uCi/ml ^{35}S-methionine at 24 hours post-infection, and th labelling continues for another 24 hours.

Neuraminidase assays. Neuraminidase activity is assayed using the fluorescent substrate 2'-(4-methylumbelliferyl)-a-D-N-Acetylneuraminic acid (Sigma) by the method of Myers, et al (R.W. Meyers, *et al.*, *Anal Biochem*, 101:166-174 (1980)). The assays are run at pH 5.1, which was shown to be optimal for the HN protein of BPI3 strain MR by Shibuta, *et al* (H. Shibuta, *et al.*, *Infect Immun*, 41:780-788 (1983)). Briefly, 225cm^2 flasks of BT cells (approximately 6x10^6 cells) are infected at an MOI of 1 with the BHV-1 viruses of interest, and infection is allowed to proceed until 100% CPE is observed (approximately 36 hours). Spinner flasks of SF9 cells (approximately 5x10^7 cells) are infected with the baculoviruses of interest at an MOI of 1 and the infection is allowed to proceed for 48 hours. After harvesting, the various harvested cell pellets are resuspended in 1.0ml cold PBS each, subjected to three freeze/thaw cycles in a dry ice/ethanol bath and then centrifuged at 12000Xg for 15 minutes at 4°C. The resulting supernatants are adjusted to 1mg/ml using the BCA protein assay (Pierce, Rockford, IL). Protein samples (50ug each) are then incubated with 80nmole of substrate in a 100ul total volume of 50mM NaAcetate (pH 5.1) at 37°C for 0-60 minutes. The reactions are stopped by the addition of 3.0ml 250mM glycine (pH 10.4), and the fluorescence intensity can be read on a Perkin Elmer LS50-B luminescence spectrophotometer (excitation 360nm, 10.0nm slit width, emission, 440nm, 2.5nm slit width), or other appropriate instrument. The sodium salt of 4-methylumbelliferone (Sigma) can be used as the standard, and under the above conditions results in a linear range between 0.2 nmoles and 10 nmoles.

Immunological methods. For immunoprecipitations, ^{35}S-methionine labelled infected or mock infected cells are solubilized in RIPA buffer (10mM Tris-HCl (pH7.5), 150mM NaCl, 0.1% SDS, 1% TritonX-100, 1% NaDeoxycholate, and 1mg/ml ovalbumin) for 1 hour at room temperature, and then the insoluble fraction is removed by centrifugation at 15Kxg for 10 minutes at RT in a microcentrifuge. The cleared lysates are then preadsorbed with 100ul of protein A Sepharose 4B (Pharmacia, 100 mg/ml in Tris-buffered saline, pH 8.0) by rocking for 1 hour at 4°C. After removing the preadsorbtion matrix the lysates are mixed with appropriate amounts of antiserum (usually 20-25ul undiluted) and incubated with rocking at 4°C for 2 hours.

To each sample, 50ul f protein A sepharose is added and rocking at 4°C is

continued for anoth r hour. The immune complexes thus adsorbed to the protein A sepharose are washed three times with HO buffer (10mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 1%NP40, and 0.5% Na deoxycholate), and then resuspended in 50ul SDS sample buffer with 2-mercapatoethanol (Sigma) and boiled for 5 minutes to dissociate the immune complexes from the matrix. The samples are resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

Examples

The BPIV-3 HN gene is expressed in two heterologous systems; a recombinant herpesvirus and a recombinant baculovirus are described. The recombinant proteins are very similar to native HN with respect to antigenicity and electrophoretic mobility, and they retain the neuraminidase activity expected for native HN, as illustrated in the following examples.

EXAMPLE 1. ***Introduction of the BPIV-3 HN into BHV-1, and selection of recombinant viruses.*** A map of the BPIV-3 HN cDNA clone and its insertion into the BHV-1 shuttle vector is shown in **FIGURE 1.** For cloning into the BHV-1 vector, remove the HN gene from the cDNA cloning vector with HindIII and BamHI (this fragment includes the 3' poly A tail retained from the cloning of the cDNA, and also includes a small portion of the SV40 polyadenylation signal region present in the vector). The HindIII site is 80bp upstream of the ATG initiation codon, **FIGURE 1.**

After filling in both the BamHI and HindIIII-generated protruding single stranded ends of the insert, ligate the HN gene into the likewise filled in HindIII site of pHAS4DBXex-1. Following transformation and selection of insert containing plasmids, determine the proper orientation of the insert relative to the CMV promoter by restriction mapping of asymmetric sites within the HN gene and the vector.

The linearized BHV-1 shuttle vector containing the BPIV-3 HN gene is allowed to recombine with the wild type BHV-1 tk locus via cotransfection into BT cells of the plasmid and unit length BHV-1 Strain "Iowa" DNA. "Iowa" is a highly virulent disease isolate strain of BHV-1. In addition, plasmid pHAS4DBX (which contains a deleted version of the BHV-1 tk gene with no inserted promoters or genes) is cotransfected with BHV-1 Iowa to obtain a tk-deleted control virus without an inserted gene. Tk-negative progeny from these cotransfections are selected and isolated by passage on RabBU cells in the presence of BDUR. The DNA from these viruses is isolated for restriction digestion and Southern hybridization using either the BPIV-3 HN gene r pHAS4 as probes (data not shown). From th BDUR

resistant transfection progeny, two BHV-1 viral clones that hybridized independently with the HN gene, HN16 and HN21 were isolated. This latter virus is referred to in the animal studies (see "utility of th invention", below) as BHVDtk HN. Several BDUR-resistant transfection progeny that contained the tk-deletion as assessed by a drop in size of the pHAS4 fragment in these clones were also isolated. A representative virus was isolated and called IowaDtk (referred to in the animal studies as BHVDtk).

EXAMPLE 2. *Introduction of the BPIV-3 HN into baculoviurs, and selection of recombinant viruses.* For expression in the BV system, we cloned the HN gene into the polyhedrin-based transfer vector pVL1392. **FIGURE 2** shows the insertion of HN into this vector. The HN gene is digested with HindIII, the single stranded protruding end is filled in with Klenow, and then the insert is excised from plasmid vector pSP72 by digestion with BamHI. Plasmid pVL1392 is digested with BglII, and the ends are filled in with Klenow fragment, then the plasmid is digested with BamHI. The HN insert is ligated into the digested pVL1392 vector via one blunt end and one sticky end, assuring the proper orientation for expression. The HN gene, properly cloned into pVL1392, is allowed to recombine into the AcNPV genome via cotransfection with BaculoGold DNA into SF9 cells.

EXAMPLE 3. *Demonstration of expression and biological activity of the HN protein in BHV-1 and Baculovirus.* BT cells infected with virus HN21 and HN16 and SF9 cells infected with baculo-HN expressed high levels of HN protein as determined by radioimmunoprecipitation. Metabolically labelled infected cell lysates were incubated with polyclonal bovine antiserum against BPIV-3 (obtained from NVSL, Ames, IA). As shown in **FIGURE 3**, HN21, HN16 (lanes 3 and 4) as well as a representative viral clone purified from the BV/HN transfection (lane 6, designated baculoHN) expressed large amounts of an approximately 70KD BPIV-3 immunoreactive protein that appeared to comigrate with native BPIV-3 HN (Lane 2). This protein was not present in wild-type BHV-1-infected (lane 1) or in mock-infected BT cells (data not shown), nor was it present in SF9 cells infected with an irrelevant recombinant baculovirus (lane 5).

The abundance of HN antigen expressed in BHV-1 and Baculovirus made it difficult to exactly pinpoint the sizes of these glycoproteins relative to native HN. Subsequent radioimmunoprecipitations using less BHV-1 and Baculovirus-expressed material, however, suggested that the BHV-1 HN protein indeed comigrated with the native HN protein, while th baculo-expressed material migrated slightly more

rapidly in SDS-PAGE (data not shown). Although we did not undertake any sort f glycoconjugate analysis, we would expect the PI-3 and BHV-1-expressed proteins, propagated in the same cell lin , to be modified similarly. The smaller size of the baculo-expressed material is easily explained, as insect cells, while recognizing and modifying N-linked glycosylation sites, do not synthesize the complex oligosaccharides observed in vertebrate cells.

To test the biological activity of the BPIV-3 HN protein as expressed in BHV-1 and BV, we assayed infected-cell lysates for neuraminidase (NA) activity. Viruses HN21 and baculoHN both displayed significant NA activity when as measured by hydrolysis of 2'-(4-methylumbelliferyl)-a-D-N-Acetylneuraminic acid by infected-cell lysates, FIGURE 4. This activity was not present in the negative controls, consisting of the BHV-1 IowaDtk virus, and a recombinant baculovirus expressing BHV-1 gIII protein.

We could not detect NA activity in the SF-4 BPIV-3 strain we used as a positive control under the conditions we used to isolate viral proteins and assay for NA. Strain MR is known to have significantly higher NA activity relative to other BPIV-3 strains (H. Shibuta, et al., *Infect Immun*, 41:780-788 (1983)), which could explain the high levels of NA activity in our recombinants relative to SF-4. In fact, previous studies show that it is very difficult to detect NA activity in some BPIV-3 strains (H. Shibuta, *et al.*, *Infect Immun*, 34:262-267 (1981)). We can conclude from these results, however, that HN21 and BVHN express biologically active HN protein.

Utility of the Invention

This invention is intended to provide the user with an effective vaccine for prevention of BPIV-3 and BHV-1 caused disease. The importance of BPIV-3 as a pathogen is its association with Bovine Respiratory Disease Complex (BRDC).

The vaccine of this invention is created with the intention of treating disease, preferably through prevention. By prevent or prevention applicant means to keep the host from developing symptoms of the disease or to mitigate the effects of the disease, that is to avert the typical diseased state. Prevention implies decisive action to stop, impede or delay the onset of disease. Prevention can include the following concepts: to hinder, frustrate, to obstruct; to intercept, possibly prohibit, impede or preclude. Preclude would suggest the onset of the disease state either does not occur or the disease pathogen is largely ineffectual in causing the disease state. Prevent r prevention can indicate th disease state is forestalled, meaning that anticipatory action to prevent or hinder the disease has occurred but the

conditions creating the disease have not been eliminated.

The usefulness of this invention is illustrated by the ability of the vaccine to provide effective protection against BPIV-3 disease.

Vaccination for this diseas includes eliciting strong neutralizing antibody responses to the HN glycoprotein. This invention consists of different embodiments that include a recombinant baculovirus and a recombinant bovine herpesvirus. These recombinant viruses express the HN protein from bovine parainfluenza virus-3, thus conferring a protective effect in the vaccinated animals.

Eight to twelve week old colostrum deprived calves were housed in separate containment facilities during vaccination and brought together as one group prior to challenge. Prior to vaccination animals were tested to make sure that they were free of both BVDV and BPIV-3 antibodies. Nineteen animals were divided into five groups. Group 1 (three animals) received the BHVDtk control virus, group two (five animals) received the BHVDtk-HN virus, group 3 (five animals) received SF9 cells infected with the baculo-HN virus, group 4 (three animals) received a SF9 cells infected with an negative control baculovirus, and group 5 (three animals) served as a non-vaccinated control. Animals received $2x10^7$ Sf9 cells infected 72 hours previously with recombinant or control baculoviruses on two separated occasions 28 days apart. Inoculations in 2 ml volumes were administered subcutaneously. BHV-1 viruses were given intranasally in 2 ml volumes at the rate of 10^6 TCID50/calf on two separate occasions, 28 days apart. All animals were challenged on day 42 post vaccination with $4X10^7$ BPIV-3 administered intranasally. Animals were bled on days 21 and 42 post-vaccination to evaluate the levels of serum neutralizing antibodies against BPIV-3. Post-challenge, vaccine efficacy was assessed by measuring the duration and quantity of challenge virus shedding in nasal secretions (isolated from the animals for 14 days post-challenge using sterile cotton swabs).

No adverse signs to vaccination were noted. **TABLE I** shows the reciprocal 50 % serum neutralizing titers of animals vaccinated with various preparations. Animals 2481, 2478, and 2489 were vaccinated with a BHV-1 control vaccine. Animals 2472, 2493, 2479, 2462, and 2435 were vaccinated with the recombinate BHV-HN virus. Animals 2477, 24487, 2476, and 2494 were vaccinated with the recombinate Baculo-HN virus. Animals 2488, 2480, and 2482 acted as Baculo virus controls. Animals 2152 and 2175 acted as non vaccinated controls. Animals vaccinated with the HN recombinant viruses exhibited increased levels of serum neutralizing antibodies to BPIV-3, demonstrating the immunogenicity of both embodiments of this invention. Animals also showed little or no signs of clinical

disease after challenge. The animals vaccinated with the HN recombinant viruses claimed in this invention were also protected from challenge, as shown by the durations of challenge virus shedding. **TABLE 2.** Virus shedding is illustrated by shading within squares for each day post challenge, and the percentage of virus excretion over both 7 and 14 days is illustrated in the right two columns of the table. Challenge virus from animals in the three control groups, BHV$_{\Delta}$tk, baculo control and non-vaccinated controls, was isolated in 86 and 95% of samples taken over the first 7 days with isolation rates from the recombinant baculo of 25% and the recombinant herpes of 74%. This marked reduction in isolation rates is again observed for the 14 day isolation rates. As well as reduction in rates of virus isolation, the quantity of challenge virus found in nasal secretions was also reduced compared to controls in the calves vaccinated with the BHV-1Dtk HN and baculo HN viruses (data not shown).

Complete Description of the Figures

Figure 1. Construction of shuttle vector for expression of the BPIV-3 HN gene (and other genes) in the BHV-1 tk locus. pHAS4 is a DNA fragment from the BHV-1 genome. The gene and gene fragments encoded in the fragment are noted. pHAS4DBX depicts the 424bp deletion made in the tk gene. The various cassettes inserted into pHAS4DBX are labelled. pHAS4DBXex1 was made by the insertion of the CMV-IE promoter and the bovine growth hormone polyadenylation signal into the tk deletion. In pHAS4DBXex1::HN (bottom) the direction of transcription of HN is noted by an arrow.

Figure 2. Construction of a recombinant baculovirus transfer vector for expression of the BPIV-3 HN gene in the baculovirus expression system. TOP: pVL1392, a polyhedrin promoter-based transfer vector for the expression of foreign genes. BOTTOM: Insertion of the HN gene into this vector.

Figure 3. Immunoprecipitations of infected-cell lysates showing expression of BPIV-3 HN from BHV-1 and baculovirus. The immunoprecipitates were brought down with a polyclonal antiserum (from calf) against BPIV-3. Lanes 1-4 were metabolically labelled infected (or mock infected) BT cells, and lanes 5 and 6 were metabolically labelled infected SF-9 cells. Lane 1: mock infected cells, lane 2: BPIV-3 strain SF4 (the HN protein runs at approximately 70kD), lane 3: HN16 (recomb. BHV-1), lane 4: HN21 (recomb. BHV-1), lane 5: control baculovirus (irrelevant recombinant), and lane 6: baculoHN.

Figure 4. HN activity in the BHV-1 and bacul virus recombinants. The

graph shows nzymatic release of 4-methylumbelliferone (4-MUBF) over time from the 2'-(4-methylubelliferyl)-a-D-N-acetylmeruaminic acid substrate, as catalyzed by freeze-thaw lysates of the various viral preps. The X axis is tim in minutes. The Y axis is nmoles of 4-MUBF released. **KEY**: Open squares, HN21 (BHVDtk-HN); closed diamonds, baculoHN; open circles, BHVDtk; closed squares, baculo-gC (a recombinant baculovirus expressing the gC protein of BHV-1, used here as an negative control); closed circles, BPIV-3. The nmoles of 4MUBF released was calculated from a standard curve of 4-MUBF concentration versus fluorescence intensity (r=0.999).

Deposit of Genetic Materials.

The present invention is not to be limited in scope by the deposited materials or the embodiments disclosed herein which are intended as single illustrations of one aspect of the invention and many of which are functionally equivalent and are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the claims.

It is also to be understood that all base pair and amino acid residue numbers and sizes given for nucleotides and peptides are approximate and used for the purposes of description.

All documents cited herein are incorporated by reference.

One skilled in the art should be able to reconstruct all the various embodiments of this invention by utilizing only the written description. However, for the sake of completeness, to ensure enablement, and to provide every opportunity for others to make and use this invention, certain genetic constructs of this invention have been deposited at recognized depositories in accordance with the Budapest Treaty.

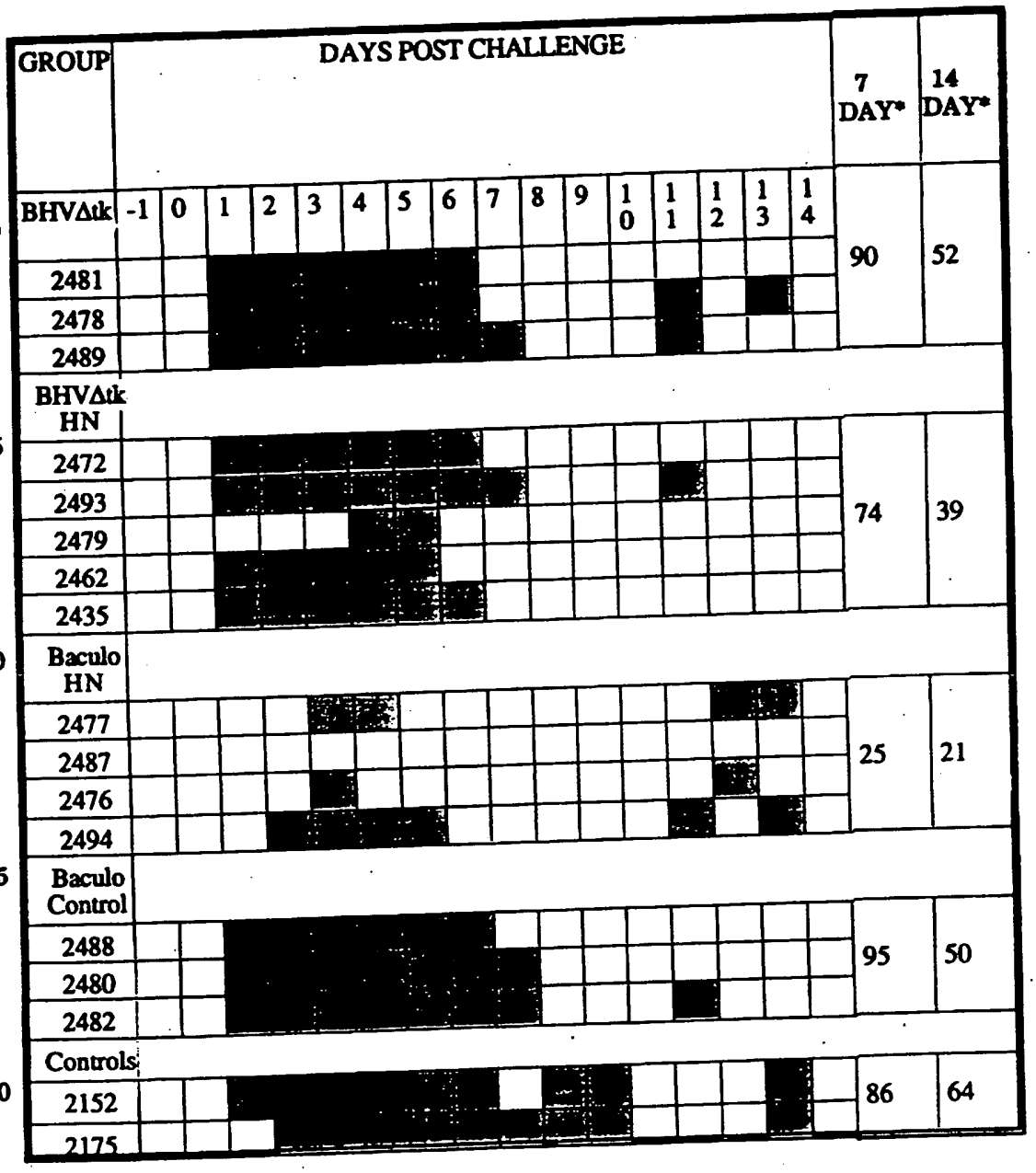
A virus was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, zip code 20852, USA. That deposit was designated UC VR-59 by the Upjohn Company and given the following number by the depository, ATCC No. VR 2478, it corresponds to the virus described herein as "HN21", or "BHVDtk HN". This deposit was received by the American Type Culture Collection depository on 14 September 1994.

TABLES

TABLE I

	Pre-Bleed	Pre-BOOST	Pre-Challenge
2481	<1:2	<1:2	<1:2
2478	<1:2	<1:2	<1:2
2489	<1:2	<1:2	<1:2
2472	<1:2	1:8	1:32
2493	<1:2	1:12	1:32
2479	<1:2	1:12	1:128
2462	<1:2	<1:2	1:24
2435	<1:2	<1:2	1:12
2477	<1:2	1:48	>1:256
2487	<1:2	1:12	1:256
2476	<1:2	1:24	>1:256
2494	<1:2	1:12	>1:256
2488	<1:2	<1:2	<1:2
2480	<1:2	<1:2	<1:2
2482	<1:2	<1:2	<1:2
2152			<1:2
2175			<1:2

TABLE II



* % EXCRETION

CLAIMS

We claim

1. A replicating nonpathogenic virus, comprising a gene or gene combination ncoding a Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in any appropriate locus of bovine herpesvirus type 1 (BHV-1).

2. A virus of claim 1, comprising the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein from bovine parainfluenza virus type 3 (BPIV-3).

3. The virus of claim 2, comprising the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in the thymidine kinase locus of BHV-1.

4. A vaccine for the prevention of diseases caused by bovine parainfluenza virus type-3, comprising a replicating nonpathogenic virus containing a gene or gene combination that encodes a Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in any appropriate locus of bovine herpesvirus type 1 (BHV-1).

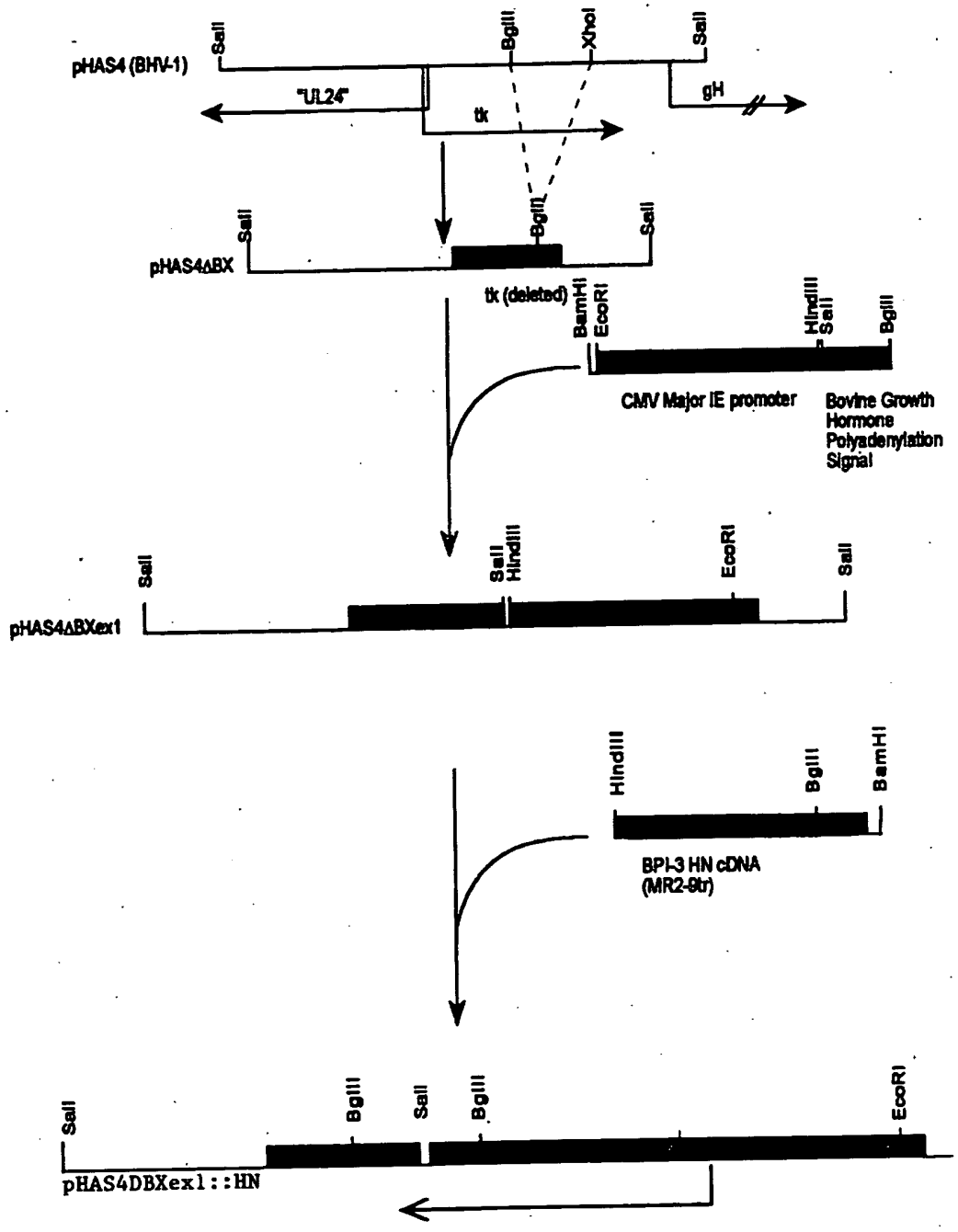
5. A vaccine of claim 4, comprising the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein from bovine parainfluenza virus type 3 (BPIV-3).

6. A vaccine of claim 5, comprising the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in the thymidine kinase locus of BHV-1.

7. A vaccine, comprising a gene or gene combination encoding a bovine Hemagglutinin/Neuraminidase (HN) glycoprotein inserted into and expressed in the baculovirus expression system.

8. A vaccine of claim 7, comprising the gene or gene combination that encodes the Hemagglutinin/Neuraminidase (HN) glycoprotein from bovine parainfluenza virus type-3 (BPIV-3).

FIGURE 1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SalI
BglII
XhoI
SalI
"UL24"
tk
gH
pHAS4ΔBX
SalI
BglII
SalI
tk (deleted)
BamHI
EcoRI
HindIII
SalI
BglII
CMV Major IE promoter
Bovine Growth Hormone Polyadenylation Signal
pHAS4ΔBXex1
SalI
SalI
HindIII
EcoRI
SalI
HindIII
BglII
BamHI
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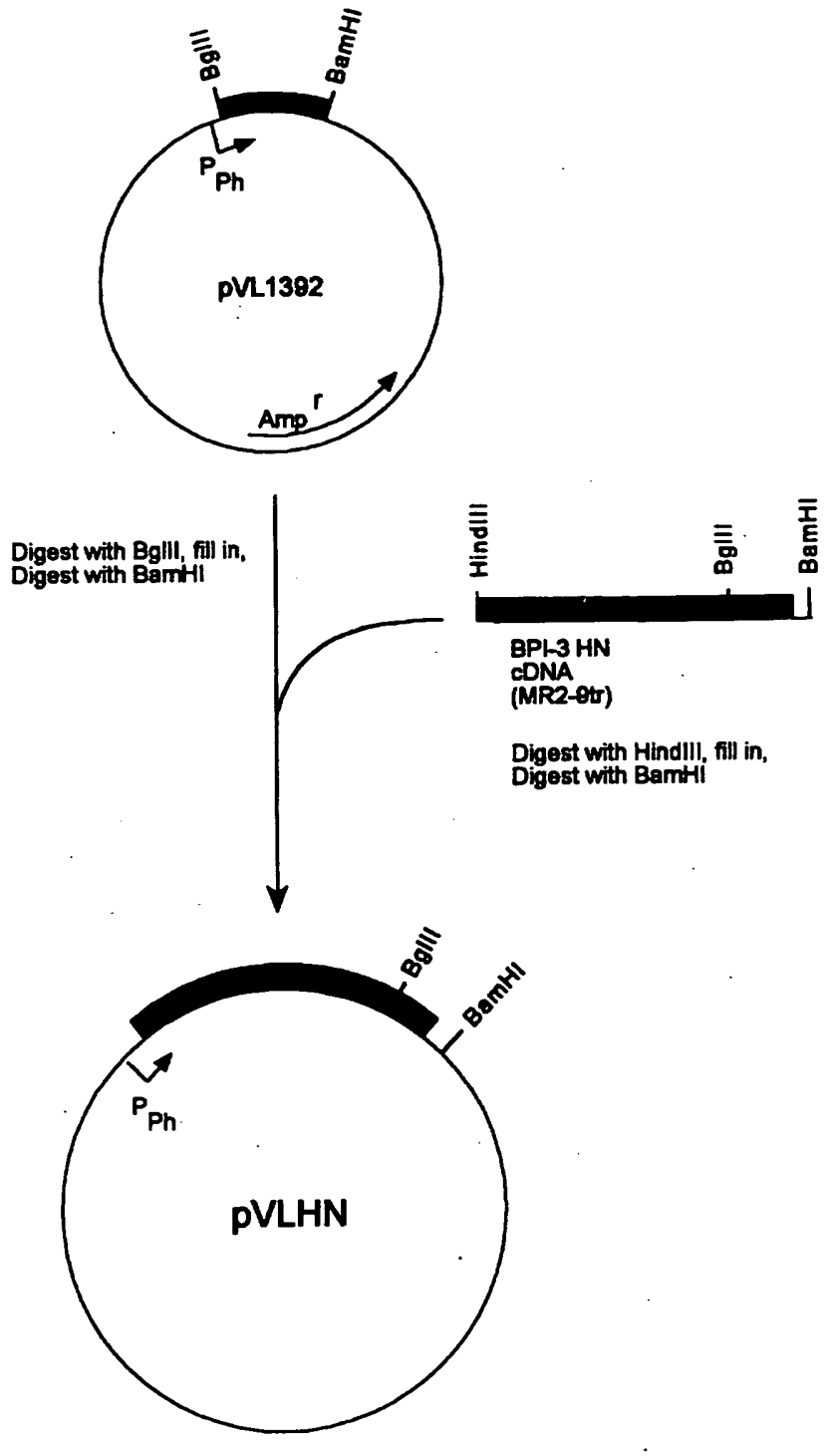
pHAS4DBXex1::HN
SalI
BglII
SalI
BglII
EcoRI

FIGURE 2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BamHI
P Ph
pVL1392
Amp r
Digest with BglII, fill in,
Digest with BamHI
HindIII
BglII
BamHI
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(MR2-9tr)
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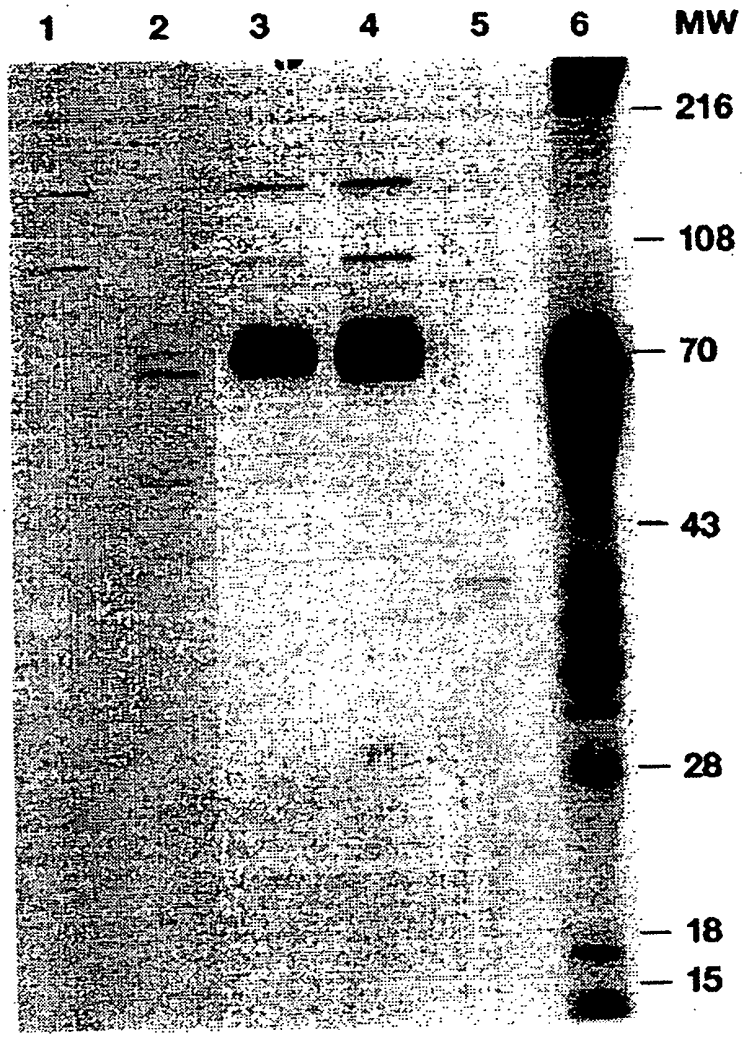
1
2
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6
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43
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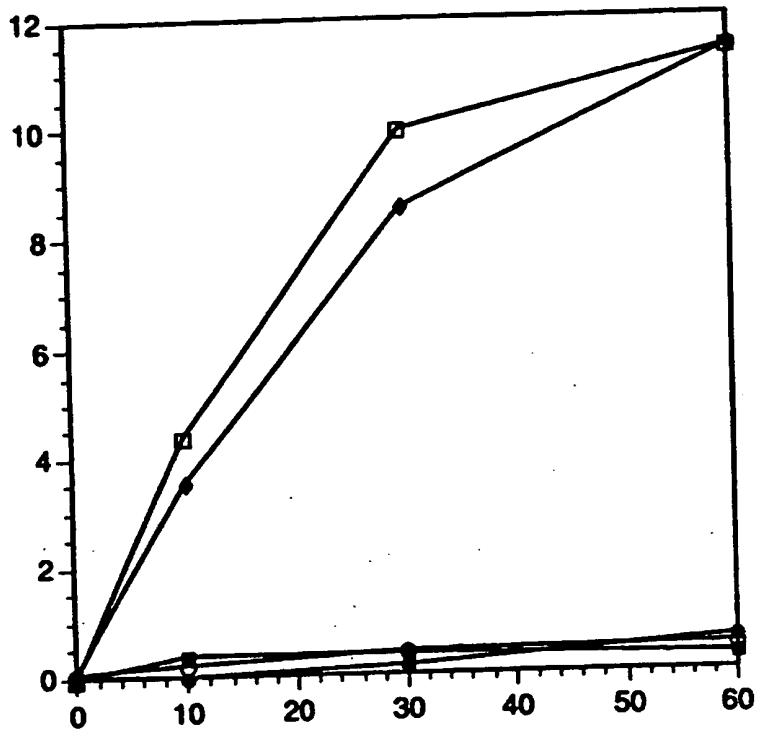
15

FIGURE 4



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/13482

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/45 A61K39/155 A61K39/245

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PATENT ABSTRACTS OF JAPAN vol. 013, no. 107 (C-576), 14 March 1989 & JP,A,63 283578 (HIROSHI SHIBUTA), 21 November 1988, see abstract ---	1-6
Y	WO,A,94 24296 (UNIVERSITY OF SASKATCHEWAN) 27 October 1994 see page 24 ---	1-6
Y	GB,A,2 161 814 (W.R.GRACE & CO.) 22 January 1986 see page 1 ---	7,8
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 April 1996	16. 04. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VIROLOGY, vol. 160, 1987, ORLANDO US, pages 465-472, XP002000517 K.L.VAN WYKE COELINGH ET AL.: "Expression of biologically active and antigenically authentic parainfluenza type 3 virus hemagglutinin-neuraminidase glycoprotein by a recombinant baculovirus" see the whole document -----	7,8

INTERNATIONAL SEARCH REPORT

Inter ional application No.
PCT/US95/13482

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1-6: A virus or vaccine, comprising a gene encoding a HN glycoprotein inserted and expressed in bovine herpesvirus type 1 (BHV-1)
- claims 7-8: A vaccine comprising a gene encoding a HN glycoprotein expressed in the baculovirus expression system

./..

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US95/13482

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

LACK OF UNITY OF INVENTION

The application is regarded to concern the following inventions:

1. Claims : 1-6 A virus or vaccine, comprising a gene encoding HN glycoprotein inserted and expressed in bovine herpesvirus type 1 (BHV-1).

2. Claims: 7 and 8 A vaccine comprising a gene encoding a HN glycoprotein expressed in the baculovirus expression system.

The problem addressed by the present invention is the provision of vaccines for bovine respiratory disease complex (BDRC). The solutions hereto provided by the present application consists of recombinant virus systems based on bovine herpesvirus type 1 (BHV-1) or baculovirus, expressing the bovine parainfluenzavirus type 3 Hemagglutinin/Neuraminidase (HN) gene.

These solutions may , **a priori**, be regarded as satisfying the requirements of unity of invention in which the presence of the HN gene provides the common special technical feature. However, JP-A-63-2835789 discloses already a solution to the above problem, i.e. a recombinant vaccinia virus with a DNA insert in a non-essential region, encoding the HN gene from bovine parainfluenzavirus type 3.

In view of the fact that recombinant viruses encoding a HN gene have been disclosed in the prior art, due to the essential difference between the BHV-1 expression system that itself is associated with BDRC on the one hand and the baculovirussystem which is an insect virus on the other hand, and due to the fact that no other technical feature can be distinguished which defines the contribution each of the claimed inventions makes over the prior art, there is no single concept underlying the claimed inventions in the meaning of Rule 13.1 of the PCT.

However, since no major additional effort was required a complete search has nevertheless been performed.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/13482

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9424296	27-10-94	NONE	
GB-A-2161814	22-01-86	US-A- 4743553	10-05-88
		AU-B- 588238	14-09-89
		AU-B- 4441785	23-01-86
		BE-A- 902921	18-11-85
		DE-A- 3524736	30-01-86
		FR-A,B 2567905	24-01-86
		NL-A- 8502063	17-02-86
		US-A- 4847081	11-07-89